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Large scale, rapid purification of recombinant tissue-type plasminogen activator

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Recombinant tissue-type plasminogen activator (rt-PA) from cultures of a genetically manipulated Bowes melanoma cell line (TRBM6) was purified in batches of average volume 451 using an autoclavable, reusable, continuous chromatography system comprising zinc chelate-Sepharose CL4B and lysine-Sepharose CL4B. After eight successive purifications the rt-PA was ultrafiltered to yield a preparation containing 4.9 mg protein/ml and 2.7×10^6 IU/ml. Analysis by SDS-polyacrylamide gel electrophoresis followed by staining with Coomassie brilliant blue R250 showed major protein bands at $M_r = 63000$ and 65000; most of the material was in the l-chain form. The potential usefulness of a simple, rapid continuous chromatography system that can be operated under aseptic conditions is discussed.

Recombinant t-PA *Zinc chelate-Sepharose CL4B* *Lysine-Sepharose CL4B* *Continuous chromatography*

1. INTRODUCTION

Plasminogen activators are attracting considerable interest for use in the treatment of acute myocardial infarction [1–3]. The evaluation of one of these activators, tissue-type plasminogen activator (t-PA), alone, or in comparison with other second generation activators such as the anisoylated plasminogen/streptokinase activator complex (APSAC or BRL 26921 [4]), requires large supplies of purified material suitable for use in the clinic. Numerous publications have detailed purification procedures for t-PA. The most frequently used affinity matrices for this purpose are zinc chelate-Sepharose 4B and concanavalin A-Sepharose 4B [5–7], immunoadsorbents [8–10] and arginine- [8,9] or lysine-Sepharose 4B [10–12]. Unfortunately, these purification regimes often involve a concentration step between columns (e.g. [5–9]) or are time consuming, for example because of low flow rates (< 10 cm·h^{-1}) during chromatography [6,9]. Furthermore, because the support for the affinity ligand is generally non-crosslinked agarose the system cannot easily be operated aseptically. In addition, the applicability of these systems to the large-scale purification of recombinant t-PA (rt-PA) has not been evaluated.

During studies on the rt-PA molecule we developed a simple, rapid purification process utilising continuous chromatography on zinc chelate-Sepharose CL4B and lysine-Sepharose CL4B. An additional advantage of this system is that both matrices and all the buffers employed can be sterilised easily.

2. MATERIALS AND METHODS

2.1. *Materials*

The recombinant Bowes melanoma cell line, TRBM6, has been described previously [13]. Eagles minimal essential medium, newborn calf serum and Nunc culture flasks were purchased from Gibco, Paisley, Scotland. The stainless steel filter (10 μm pore size) was from Pall Process Filtration, Portsmouth, England. Zinc chelate-Sepharose CL4B [14] and lysine-Sepharose CL4B

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[15], were kindly supplied by Drs T.J. Grinter and K.T. Veal. Ultrafiltration membranes (YM10) were obtained from Amicon, Stonehouse, England. The sources of the other reagents have been described [16].

2.2. *Cell culture*

TRBM6 cells were cultured and were harvested for rt-PA as described before [16]. The serum-free harvest medium was collected aseptically into a polypropylene carboy. Cells or cell debris were removed by passage through a stainless steel filter.

2.3. *Purification*

The first purification matrix was zinc chelate-Sepharose CL4B. The column was equilibrated with 0.02 M phosphate, 0.15 M sodium chloride, 0.01% Tween 80, pH 7.4 (PBS/TW), and then the filtered harvest medium was passed through. The column was immediately washed with (i) PBS/TW, (ii) 0.02 M Tris, 1.0 M sodium chloride, 0.01% Tween 80, pH 7.4, (iii) 0.02 M phosphate, 0.3 M sodium chloride, 0.01% Tween 80, pH 7.4, and was finally eluted with (iv) 0.02 M phosphate, 0.3 M sodium chloride, 0.05 M imidazole, 0.01% Tween 80, pH 7.4. The eluate of the imidazole-containing buffer was passed directly onto a column of lysine-Sepharose CL4B that had been equilibrated previously in PBS/TW. After the equivalent of two bed volumes of the zinc chelate-Sepharose CL4B column had passed through the lysine-Sepharose CL4B column the flow from the former was stopped. The lysine-Sepharose CL4B column was then washed sequentially with (i) PBS/TW, (ii) 0.02 M Tris, 0.5 M sodium chloride, 0.01% Tween 80, pH 7.4, and (iii) 0.02 M Tris, 0.5 M sodium chloride, 0.5 M L-arginine, 0.01% Tween 80, pH 7.4. All stages of the chromatography were carried out at 5–10°C. The velocity of flow was varied as follows. The application of the harvest medium to the zinc chelate-Sepharose CL4B column was at 32 $cm \cdot h^{-1}$. All elutions were at 100 $cm \cdot h^{-1}$. The volume of affinity matrix used in each purification was on the following basis: 10 l harvest medium; 200 ml zinc chelate-Sepharose CL4B; 40 ml lysine-Sepharose CL4B. Height/diameter ratios of the columns were in the range 3–8. All buffers and affinity matrices used were sterilised (121°C/15 min).

2.4. *Concentration*

After eight successive purifications individual pools of rt-PA were thawed and mixed and were concentrated using stirred-cell ultrafiltration (YM10). The ultrafiltered retentate was stored at −40°C.

2.5. *Miscellaneous*

SDS-polyacrylamide gel electrophoresis (SDS-PAGE), fibrin zymography, a colorometric protein analysis and the assay for amidolytic activity were carried out as described previously [16]. Protein content was also determined, after acid hydrolysis of the product, by amino acid analysis using the Waters PICO. TAG system [17]. Fibrinolytic activity was measured essentially as before [18]; calibration was against the international standard for t-PA, Lot 83/517.

3. RESULTS

Serum-free harvest medium from cultures of TRBM6 cells was collected and passed batchwise through a stainless-steel filter to remove cell debris. This filtration usually took place at 50 l/h but occasionally was increased to 200 l/h apparently without detrimental effect. The rest of the purification, on the zinc chelate-Sepharose CL4B and lysine-Sepharose CL4B columns, was carried out in series. Filtered harvest medium could be pumped onto the column of zinc chelate-Sepharose CL4B at a flow rate of 200 $cm \cdot h^{-1}$. However, this application was generally carried out overnight, at a reduced velocity. The column was washed to remove contaminating proteins as described in section 2. Recombinant t-PA was desorbed from the matrix using 0.02 M Tris, 0.3 M sodium chloride, 0.05 M imidazole, 0.01% Tween 80, pH 7.4. The eluate containing the rt-PA was diverted directly onto the column of immobilised lysine. The velocity of flow was adjusted to 200 $cm \cdot h^{-1}$ (of surface area of the lysine-Sepharose CL4B column). Under these conditions virtually all the rt-PA activity adsorbed to the lysine-Sepharose CL4B column. The rt-PA was subsequently desorbed by a buffer containing 0.5 M L-arginine and the eluate was fractionated.

The most active fractions were identified using a rapid microtitre plate-based assay employing the chromogenic substrate S-2288 and were pooled

Table 1

Recovery of rt-PA from eight individual purifications, totalling 360 l harvest medium, after the various stages of the process

Solution analysed	Fibrin plate activity (IU × 10^{-6})	[%]	Protein[a] (mg)	Specific activity (IU/mg protein)
Harvest medium (pre filtration)	202	[100]	13400	15100
'Active fractions' from zinc chelate-Sepharose CL4B	143	[71]	not determined	–
Active fractions from lysine-Sepharose CL4B	169	[84]	356	475000
Ultrafiltered retentate	176	[87]	371 (319)[b]	474000 (552000)[b]

[a] Protein figures were derived using the colorimetric assay described in section 2

[b] Corresponding figures using protein content determined by amino acid analysis

The activity of the 'active fractions' from the zinc chelate-Sepharose CL4B column was based on assay of a sample collected continuously during application to the lysine-Sepharose CL4B column

and stored at −40°C. Most of the activity was in a volume equivalent to half the bed volume of the immobilised lysine column but generally the pool was made of fractions totalling twice this volume. Recovery of fibrinolytic activity during the purification stages was 70% or better (table 1).

After several purification runs individual pools were thawed and mixed and were concentrated by stirred-cell ultrafiltration using membranes with a nominal cut-off of M_r 10000. Loss at this stage was minimal (table 1). The ultrafiltered retentate had a protein content, based on amino acid analysis, of 4.9 mg/ml and, therefore, the specific activity of the rt-PA was 552000 IU/mg protein. Despite the absence of aprotinin in the harvest medium or in any of the purification buffers the rt-PA in the purified pool was mainly l-chain (fig.1). It was aliquoted and stored at −40°C for further studies. SDS-PAGE followed by fibrin zymography of the final product revealed a major band at M_r ≈63000 (not shown).

4. DISCUSSION

The treatment of acute myocardial infarction using t-PA [1] has been aided by its successful isolation on a large scale. Although several large-scale procedures for the purification of t-PA have been published [6,7,9,10] none has been particularly rapid or has used systems capable of aseptic operation. We believe that the simple and rapid continuous purification process described here, as well as being applicable to rt-PA, has significant advantages over previously described large-scale procedures. In particular, the use of crosslinked agarose as the support for the immobilised ligands allows not only both matrices to withstand autoclaving but also attainment of high flow rates. At such flow rates the overall purification procedure was rapid; this is probably one reason why there was no need, in contrast to a previous report [5], to use aprotinin in either the harvest medium or the purification buffers in order to obtain main-

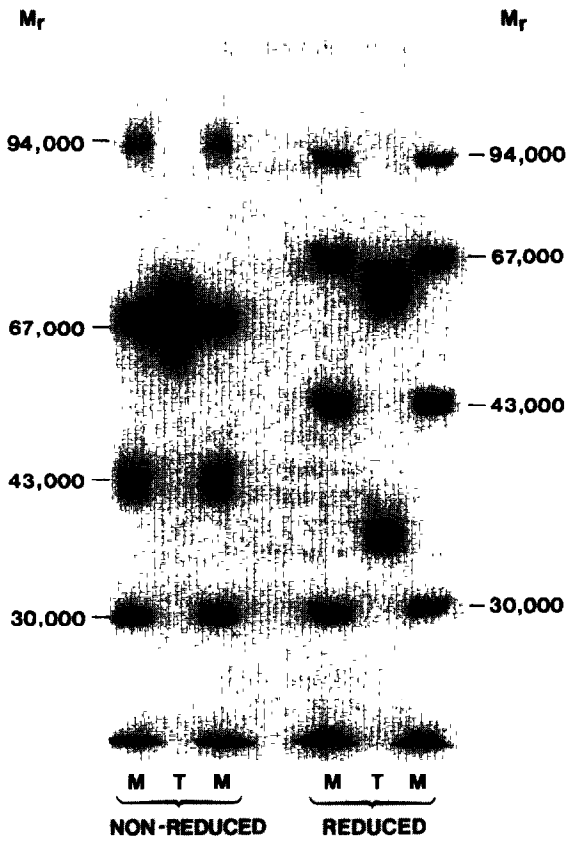

Fig.1. SDS-PAGE of purified rt-PA. Ultrafiltered retentate was diluted in 0.125 M Tris, 0.1% SDS, pH 6.8, in the presence or absence of 2-mercaptoethanol (5%, v/v) and analysed as described in section 2. The stained gel is shown. Lanes marked M are marker proteins; lanes marked T are rt-PA.

ly l-chain material. The reusability of both matrices was an additional advantage (not shown). The purity of the product determined by SDS-PAGE followed by staining for protein compared well with that from other procedures. Also, the specific activity of the product, 552000 IU/mg protein, compared with a figure of 270000 to 340000 IU/mg described elsewhere [19]. (It is more difficult to make further comparisons owing to the use by other authors of the urokinase international standard.)

Further studies on the rt-PA molecule in this laboratory have required more concentrated material. Although we have achieved this by discontinuous ultrafiltration we cannot see any reason why, on larger scale operations, the procedure could not be carried out in line. An alternative to this approach is the elution of rt-PA from lysine-Sepharose using a volatile buffer such as 0.01 M formate [20], followed by lyophilisation. In either case the requirement for further formulation into a product suitable for the clinic is likely to be minimal. The purification process described here has been in routine use for many months, providing us with significant amounts of rt-PA for research evaluation both in vitro and in vivo.

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